Silencing the *Agrobacterium tumefaciens* oncogenes, *iaaM* and *ipt*

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Abstract

Crown gall tumors result from excessive production of auxin and cytokinin in plant cells transformed by *A. tumefaciens*. High phytohormone levels result from expression of three oncogenes transferred into the plant genome from *A. tumefaciens*: auxin biosynthesis genes *iaaM* and *iaaH*, and *ipt*, a cytokinin biosynthesis gene. Tobacco containing transgenes that encode untranslatable sense-strand *iaaM* and *ipt* RNAs were resistant to crown gall. *iaaM* was silenced at a higher frequency (56%) than *ipt* (2%). Constructs that generate both sense and antisense RNA from *iaaM* and *ipt* silenced *iaaM* but not *ipt*.

To determine whether specific sequences were responsible for efficient silencing of *iaaM*, we generated sense and antisense RNA from three different 600-base-pair (bp) sections of *iaaM*. Although each construct was derived from an 1800-bp transgene that silenced *iaaM*, none of the 600-bp fragments silenced the oncogene. Shortening the transgene from 1800 bp to 600 bp may disrupt specific nucleotide sequences required to silence *iaaM*.

Introduction

A. tumefaciens causes crown gall on fruit and nut trees, grapevines, and other crops by transforming them to overproduce auxin and cytokinin. The *iaaH* and *iaaM* genes are responsible for auxin overproduction, and *ipt* causes cytokinin synthesis. These oncogenes are located within the transferred DNA (T-DNA), a region of the tumor-inducing (Ti) plasmid that integrates into the host genome (Fig. 1). *iaaM* encodes tryptophan monooxygenase, which converts tryptophan to indole-3-acetamide. Indole acetamide hydrolase, encoded by *iaaH*, converts indole-3-acetamide to indole-3-acetic acid, an auxin (1-3)(Fig. 2.). Isopentenyl-transferase, encoded by *ipt*, converts adenosine 5'-monophosphate (AMP) to isopentenyl adenosine monophosphate, a cytokinin(4). Silencing of *ipt* and either *iaaM* or *iaaH* prevents crown gall.

Post-transcriptional gene silencing (PTGS) results in degradation of mRNAs encoded by transgenes and endogenous genes with sufficient sequence identity. Double-stranded RNA is a potent PTGS inducer. In this study we compared the frequency of PTGS induction by constructs designed to express both sense and antisense oncogene RNAs with sense-only constructs.

Figure 1. ***A. tumefaciens*** **T-DNA**

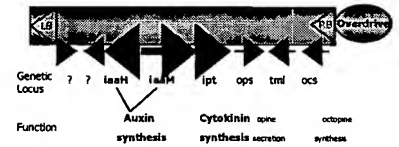
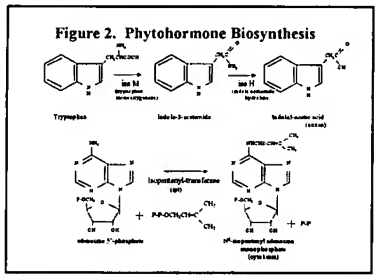


Figure 2. Phytohormone Biosynthesis



Materials and Methods

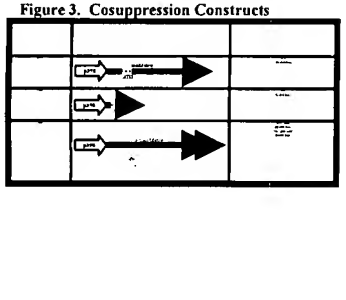
Bacterial strains: The *Agrobacterium tumefaciens* strains used in this study were: A348, wild-type with plasmid pTiA6NC (13); EHA101, with disarmed (no T-DNA) Ti plasmid, pTiBo542 (14). All gene silencing constructs used in this study were harbored in EHA101.

Mixed infection assays: *Kalanchoe daigremontiana* stems and potato discs were inoculated with *A. tumefaciens* cultures mixed in a 4-to-1 ratio of silencing strain to pathogenic strain. *A. tumefaciens* cultures were grown at 28°C overnight in YEP broth to the same cell densities (Klett 300-500). For *K. daigremontiana* inoculations, broth cultures were mixed in a 4:1 ratio. For potato inoculations, cultures were washed IX in PBS buffer, suspended in 1 volume PBS, and then mixed in a 4:1 ratio. *K. daigremontiana* stems were wounded with a sterile toothpick, and each wound was inoculated with 5µL of mixed culture. Plants were maintained in a greenhouse or growth room and scored between 6-12 weeks post-inoculation. Potato discs (red varieties) approximately 3-mm thick were cut from 7mm-diameter cores, placed on water agar, and inoculated with 5µL of mixed culture within 10 minutes of cutting (15). Discs were incubated at room temperature and scored after 3 weeks.

Results

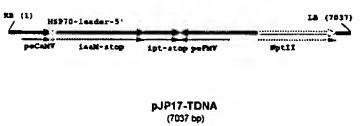
To cosuppress *iaaM* and *ipt* (5), we designed transgenes to express untranslatable sense-strand RNAs from the oncogenes (Fig. 3). Tobacco plants homozygous for these transgenes silenced *iaaM* in 56% of the lines (35 of 63), whereas *ipt* was silenced in only 2% (1 of 45 lines) (Fig. 3). This 28-fold difference occurred even when the two transgenes were fused (Fig. 3, *ipt*-stop—*iaaM*-stop). Although both transgenes were controlled by the same promoter (p35S), *iaaM* transgene mRNA accumulation was consistently low or absent, whereas *ipt* transgene mRNA accumulation was high in non-silencing lines. This difference between *iaaM*- and *ipt*-transgenic lines suggests that silencing efficiency may vary with the target sequence.

Figure 3. Cosuppression Constructs



In an attempt to silence both *ipt* and *iaaM* in a higher percentage of transgenic lines, we designed a transgene to express both sense and antisense RNA from fused *iaaM* and *ipt* sequences (Fig. 4). We developed two assays to evaluate *iaaM* silencing. *Kalanchoe daigremontiana* stems develop pronounced tumors in response to *A. tumefaciens*. These tumors have distinct morphologies corresponding to overproduction of cytokinin, auxin, or both. Smooth cytokinin-driven tumors that lack adventitious roots develop if auxin overproduction is blocked, whereas wild-type tumors are rough and produce adventitious roots (Fig. 5). Disarmed *A. tumefaciens* strains, such as EHA101, are used for plant transformation because they carry Ti plasmids containing genes for T-DNA transfer but not tumorigenesis. These strains can transfer T-DNA constructs from a binary plasmid vector into plant cells. Coinoculation of *K. daigremontiana* with wild-type *A. tumefaciens* (A348) and EHA101 carrying pJP17 (Fig. 4) (1800 bp of *iaaM* fused to *ipt* and flanked by opposing promoters) resulted in cytokinin-driven tumors, indicating that *iaaM*, but not *ipt*, was silenced (Fig. 5B). Coinoculation with wild-type *A. tumefaciens* and vector-containing EHA101 did not disrupt normal tumorigenesis by A348 (Fig. 5A). This assay involves multiple transformation events thereby masking chromosomal position effects that can influence the effectiveness of a particular integrated PTGS-inducing transgene.

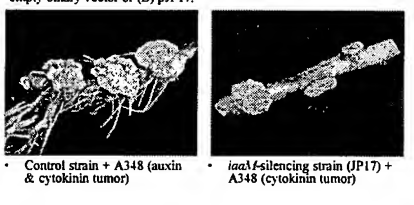
Figure 4. dsRNA ***iaaM*** **and** ***ipt*** **Construct**



pJP17-TDNA
(7037 bp)

Figure 5. Mixed Infection Gene Silencing Assay

Kalenchoe daigremontiana plants were coinoculated with wild type *A. tumefaciens* strain A348 and disarmed strain EHA101 containing either (A) empty binary vector or (B) pJP17.

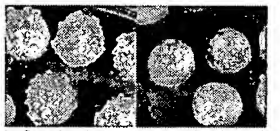


- Control strain + A348 (auxin & cytokinin tumor)
- *iaaM*-silencing strain (JP17) + A348 (cytokinin tumor)

To quantitatively evaluate the degree of *iaaM* silencing, we coinoculated potato discs with the same *A. tumefaciens* mixtures used in the *Kalanchoe* assay. Potato tubers respond to auxin but not to cytokinin. When inoculated with wild-type (or *ipt*-mutant) *A. tumefaciens*, potatoes develop individual tumor foci (Fig. 6), but potatoes do not respond when inoculated with an *iaaM*-mutant (6). PTGS-inducing constructs that silence *iaaM* prevented tumorigenesis on potato tuber discs. Four-fold fewer tumor foci formed on potato discs coinoculated with wild-type *A. tumefaciens* and *iaaM*-silencing strain, EHA101(pJP17), than on discs coinoculated with wild-type and vector-only strain (Figures 6 & 7). The silencing strain caused a statistically significant 4-fold decrease in tumor formation (Fig. 7) (p-value = 0.001 in a two-sample t-test).

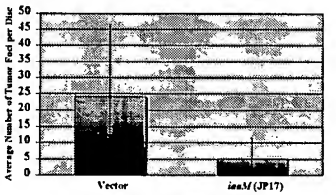
Figure 6 . Gene Silencing of ***iaaM*** **on Potato**

Potato discs were coinoculated with with wild type *A. tumefaciens* strain A348 and disarmed strain EHA101 containing either (A) empty binary vector or (B) pJP17.



A. Control strain + A348 B. *iaaM*-silencing strain (JP17) + A348

Figure 7. Silencing by ***iaaM*** **Transgene**



To examine sequence requirements for *iaaM* silencing, we tested the ability of three different 600-bp fragments derived from the highly effective 1800-bp transgene to silence *iaaM* (Fig. 8). *K. daigremontiana* developed wild-type tumors when coinoculated with A348 and these constructs, indicating that the 600-bp *iaaM* sequences did not silence *iaaM*. Likewise, potato discs coinoculated with A348 and each silencing strain developed tumor foci as frequently as those coinoculated with A348 and a vector-only control strain (Fig. 9). Truncations of 200 and 400 bp from the 3' end or 200 bp from the 5' end of the full-length *iaaM* transgene abolished its ability to silence *iaaM* (Fig. 8). However, a 400-bp truncation at the 5' end of the transgene partially silenced the wild-type *iaaM* oncogene (Fig. 10).

Figure 8. ***iaaM*** **Silencing Constructs**

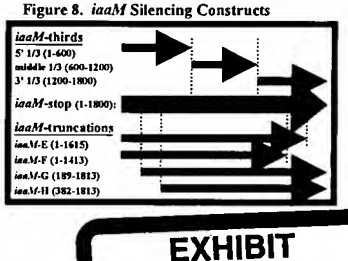


Figure 9. Silencing by ***iaaM*** **Thirds**

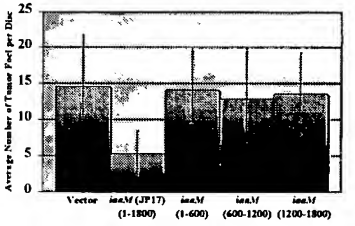
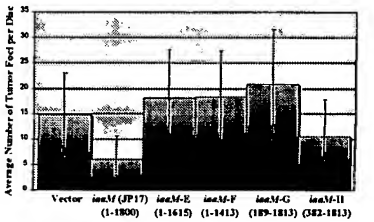


Figure 10. Silencing by ***iaaM*** **Truncations**



Discussion

We compared the silencing efficiency of several transgene constructs designed to silence the *A. tumefaciens* oncogene *iaaM*. Initial transgenes designed to generate untranslatable sense RNAs of *iaaM* and *ipt*, silenced *iaaM* at a much higher frequency than *ipt* (56% vs. 2%, Fig. 3). To improve the rate of silencing, we designed a construct to produce sense and antisense RNAs from *iaaM* and *ipt* (Fig. 4). In transient assays this construct silenced *iaaM* but not *ipt*. This was surprising in light of the breadth of genes that can be silenced by dsRNA in a variety of organisms (7-10). Perhaps there is a sequence component to silencing that is revealed when the PTGS trigger is suboptimal, such as separate sense and antisense RNAs, as opposed to hairpin RNAs (hpRNAs) that can trigger silencing at a rates of 85-100% (11,12).

The differential silencing of *iaaM* and *ipt* in our system suggests that sequence-specific factors may be involved in silencing. To explore sequence requirements for PTGS, we built constructs to express dsRNA from various sections of the *iaaM*-stop transgene that silenced *iaaM* (Fig. 8). Surprisingly, only one of these constructs, the 3' 1400 bp of the *iaaM*-stop transgene, reduced oncogene expression significantly, although to a lesser extent than the 1800-bp transgene (Fig. 10). However, the 3' 1600 bp of the transgene did not silence *iaaM*. Perhaps particular sequences, facilitate or inhibit hybridization of their complementary sense and antisense RNAs, making some sequences better PTGS inducers than others. Some sequences may also contain more self-complementarity than others, thus making more efficient trigger RNAs. It will be interesting to compare the silencing efficiencies of hpRNA constructs derived from the sequences used in this study. Perhaps hpRNAs of any part of the *iaaM* sequence will serve as strong PTGS triggers and will mask the sequence effects seen with our sense-antisense constructs.

References

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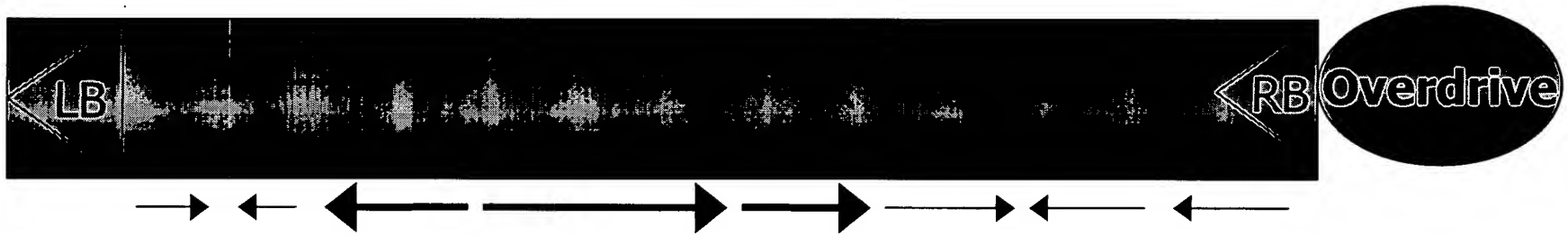
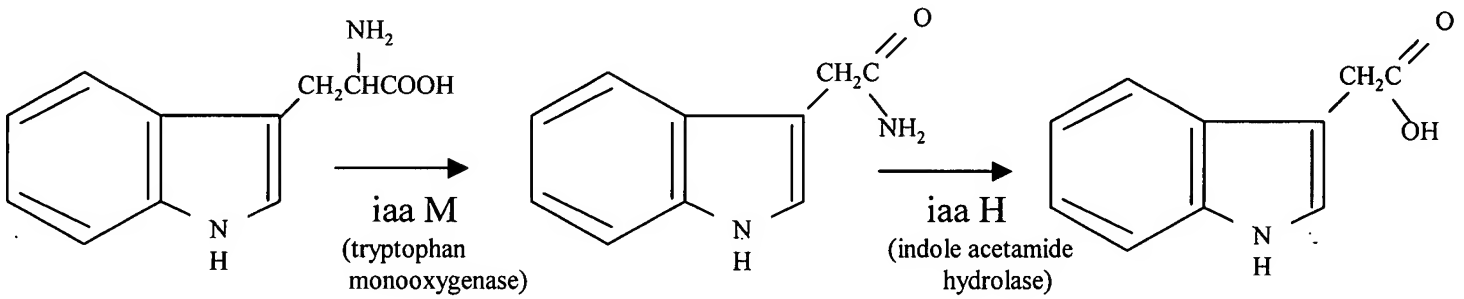
15. Pueppke, S.G. & Benny, U.K. (1981) *Physiological Plant Pathology* **18**, 169-179.

Figure 1. *A. tumefaciens* T-DNA



Genetic Locus	?	?	**iaaH**	**iaaM**	**ipt**	ops	tml	ocs
Function			**Auxin synthesis**		**Cytokinin synthesis**	opine secretion		octopine synthesis

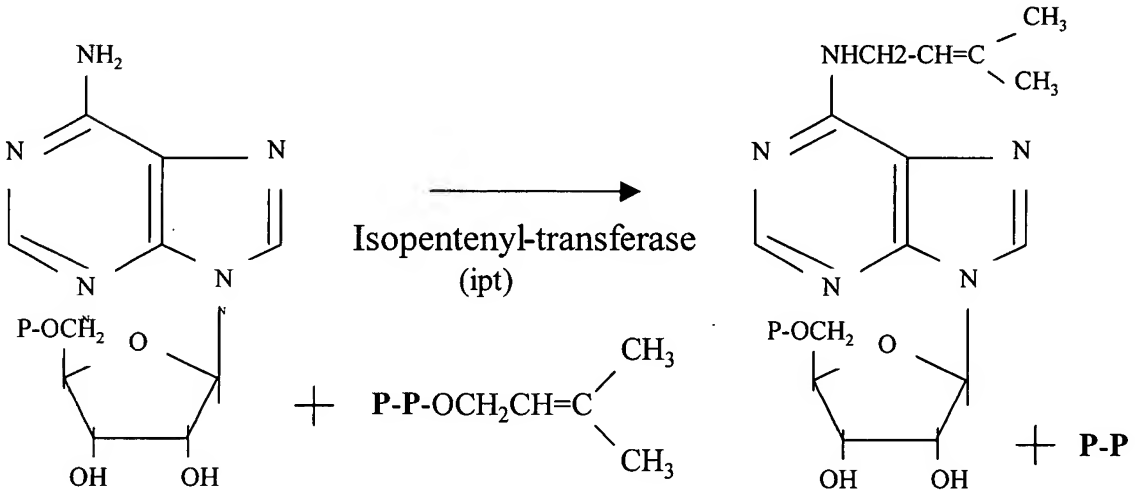
Figure 2. Phytohormone Biosynthesis



Tryptophan

Indole-3-acetamide

Indole-3-acetic acid
(auxin)



adenosine 5'-phosphate

N^6-isopentenyl adenosine
monophosphate
(cytokinin)

Figure 3. Cosuppression Constructs

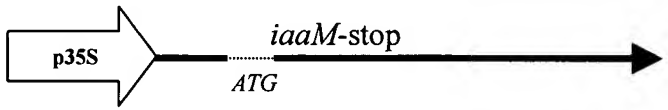
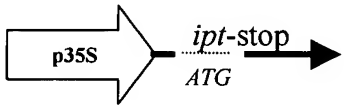
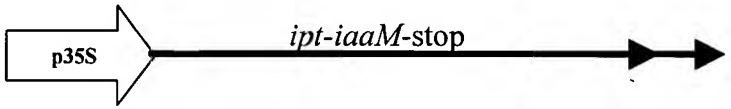
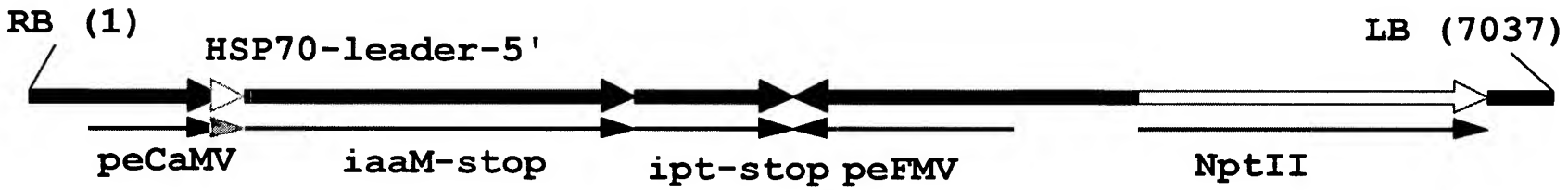
Tobacco line	T-DNA construct	Silencing rate
TDP1		56%
CW1		2%
CW4		41% - ***iaaM*** 3% - ***ipt & iaaM***

Figure 4. dsRNA *iaaM* and *ipt* Construct

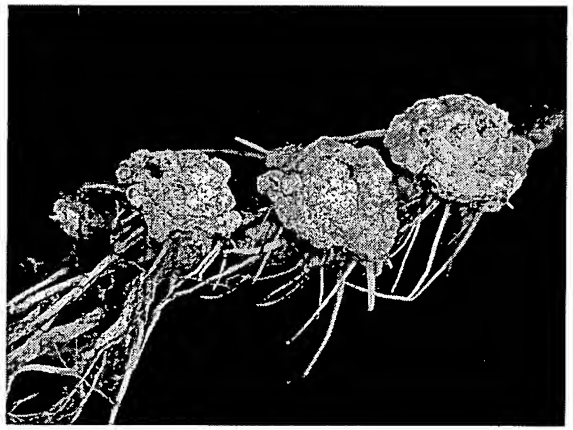


pJP17-TDNA

(7037 bp)

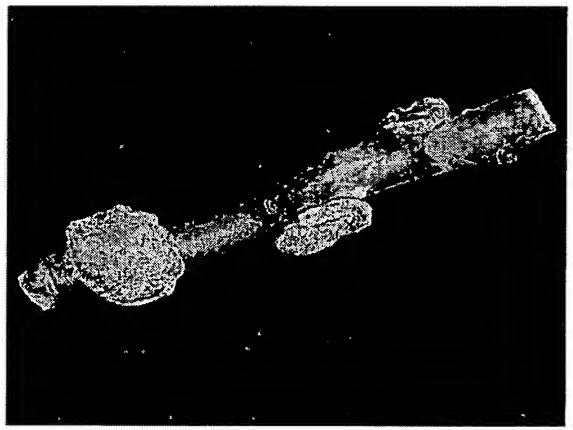
Figure 5. Mixed Infection Gene Silencing Assay

Kalenchoe daigremontiana plants were coinoculated with wild type *A. tumefaciens* strain A348 and disarmed strain EHA101 containing either (A) empty binary vector or (B) pJP17 (fig. 4).



A. Control strain + A348

(auxin & cytokinin tumor)

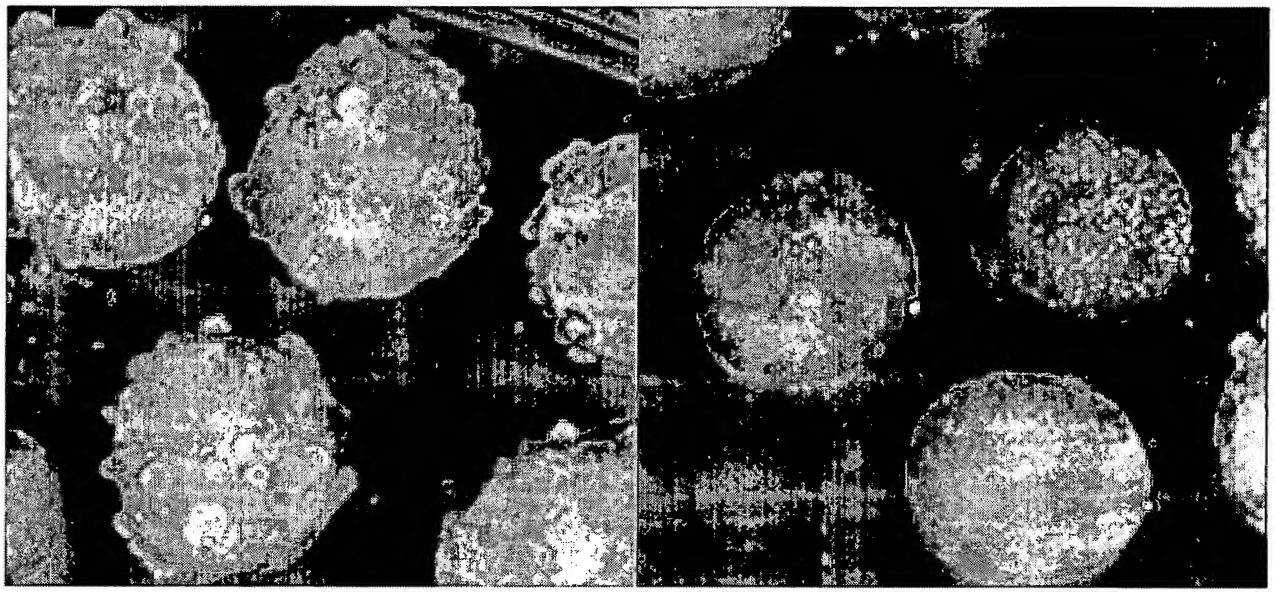


B. *iaaM*-silencing strain (JP17) + A348

(cytokinin-driven tumor)

Figure 6 . Gene Silencing of *iaaM* on Potato

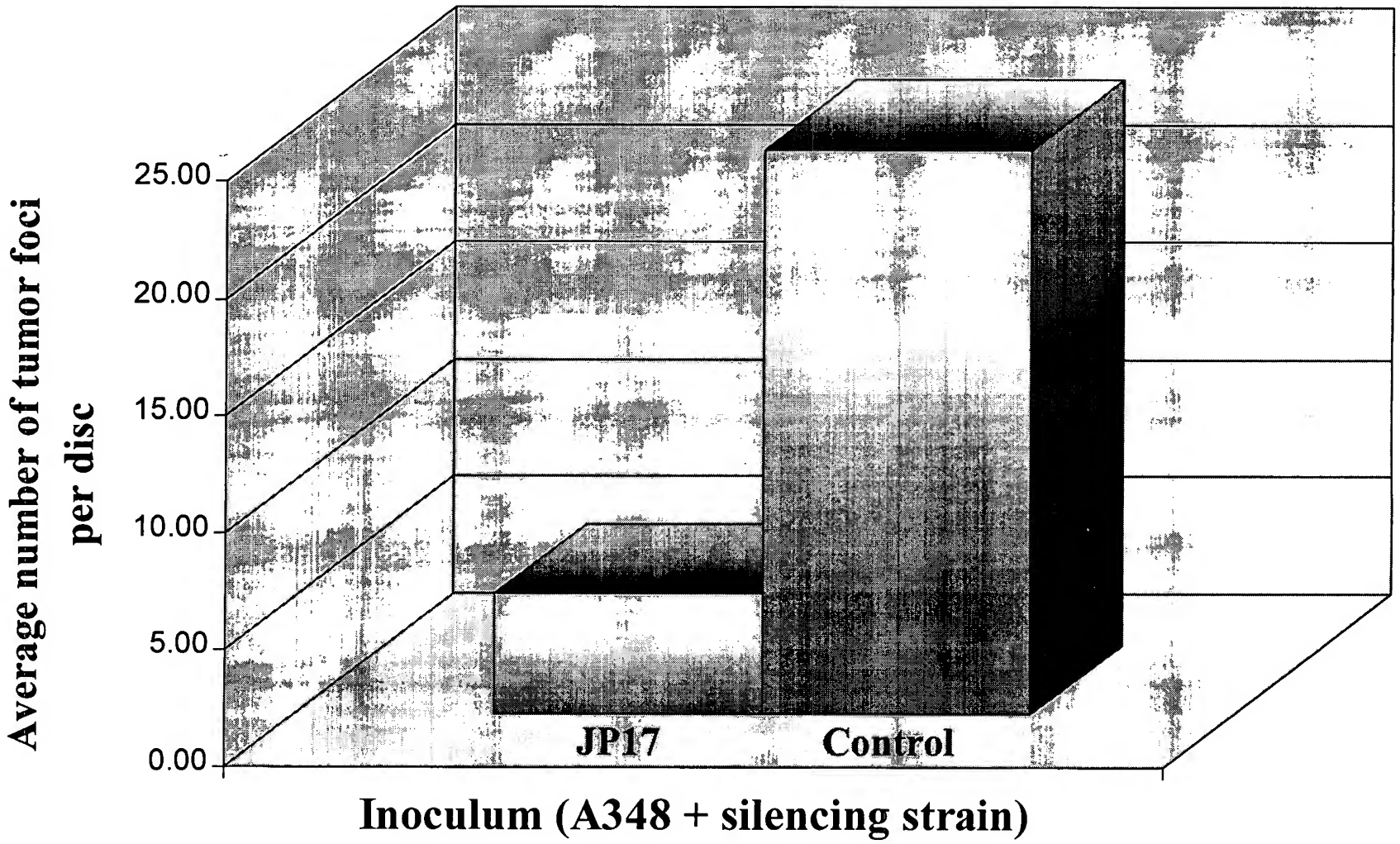
Potato discs were coinoculated with with wild type *A. tumefaciens* strain A348 and disarmed strain EHA101 containing either (A) empty binary vector or (B) pJP17 (fig. 4).



A. Control strain + A348

B. *iaaM*-silencing strain (JP17) + A348

Figure 7. Silencing by *iaaM* -stop



(Two-sample t-test p-value=0.001, 95% c.i. 8-30 foci/disc)

Figure 8. *iaaM* Silencing Constructs

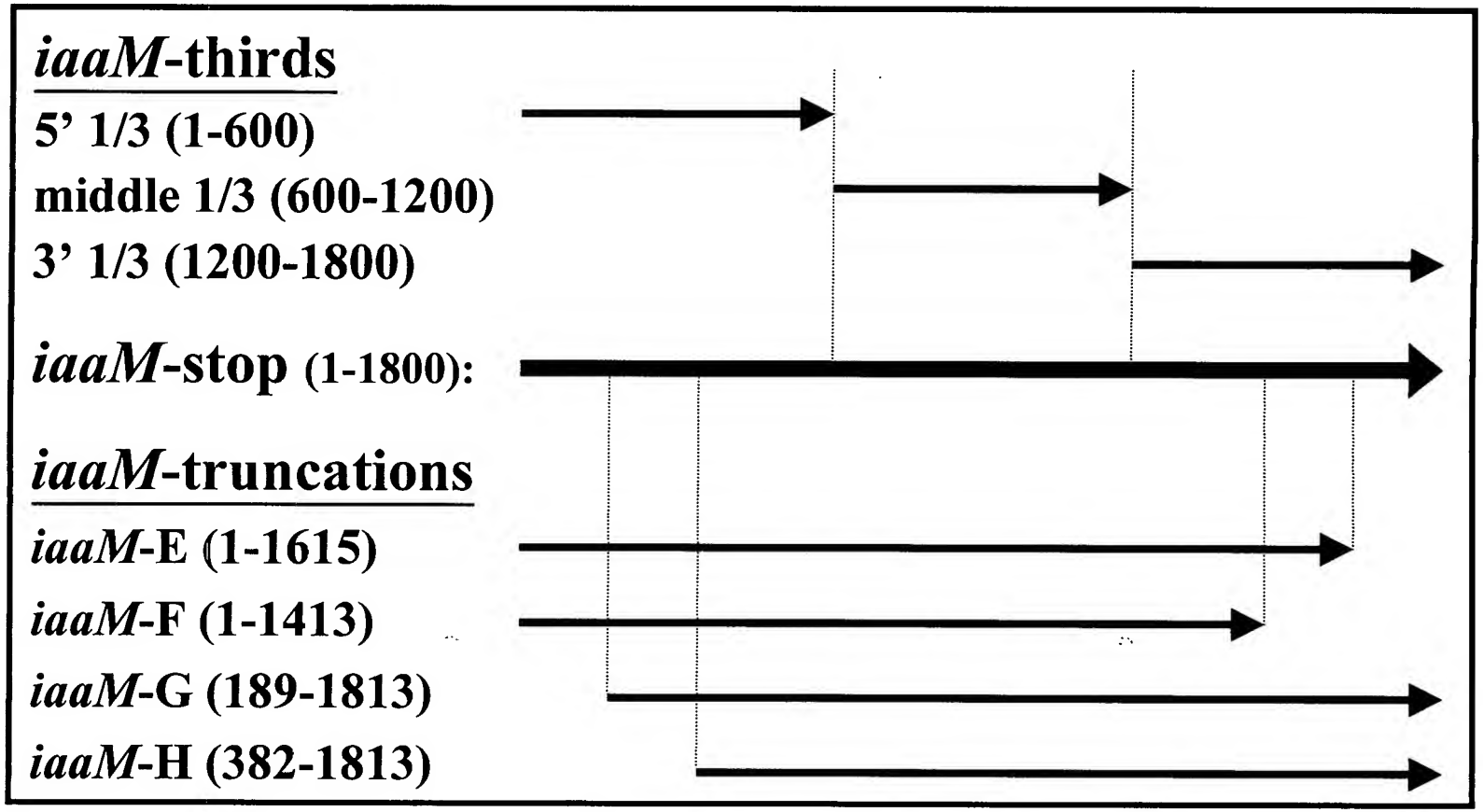


Figure 9. Silencing by *iaaM* thirds

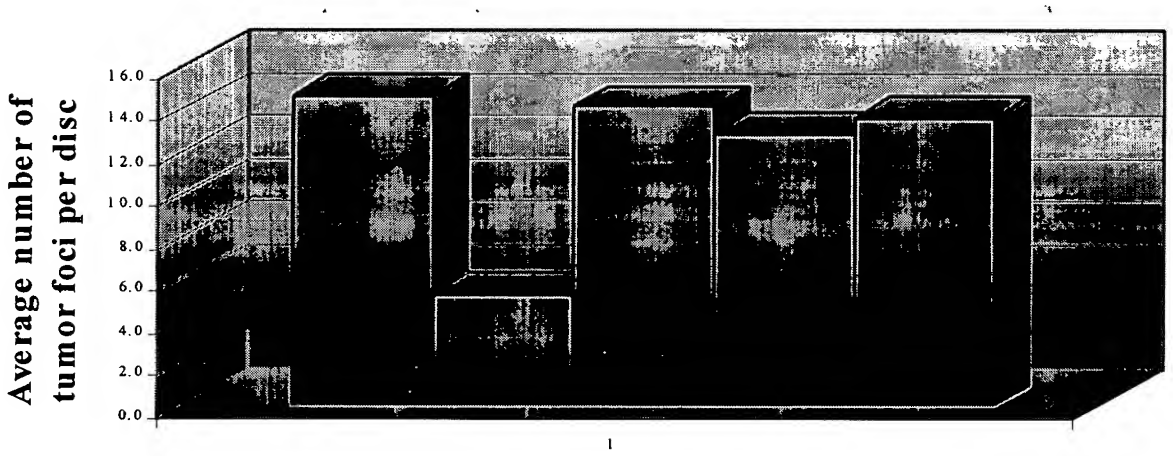


Figure 10. Silencing by *iaaM* Truncations